Expression of fatty acyl desaturase and elongase genes, and evolution of DHA:EPA ratio during development of unfed larvae of Atlantic bluefin tuna (*Thunnus thynnus* L.)

Sofia Morais[a*], Gabriel Mourente[b], Aurelio Ortega[c], Jamie A. Tocher[a] and Douglas R. Tocher[a]

[a] *Institute of Aquaculture, University of Stirling, FK9 4LA Stirling, Scotland, UK*

[b] *Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain*

[c] *Planta Experimental de Cultivos Marinos, Instituto Español de Oceanografía (IEO), 30860 Puerto de Mazarrón (Murcia), Spain*

* Corresponding author: Tel.: 00441786467993; fax: 00441786472133

E-mail address: sofia.morais@stir.ac.uk

ABSTRACT

The concentration of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) in neural tissues is known to be crucial for effective prey capture from the time of first feeding in marine fish larvae. Furthermore, tissues of tunids, including Atlantic bluefin tuna, have relatively high levels of DHA (docosahexaenic acid, 22:6n-3) and a high ratio of DHA:EPA (eicosapentaenoic acid; 20:5n-3) compared to most other species. Although the lipid biochemistry underpinning the high DHA:EPA ratio in tuna is unclear, it has been generally assumed that they must selectively accumulate and retain DHA in their tissues. In the present study, we investigated lipid and fatty acid metabolism during early development of Atlantic bluefin tuna and determined the changes in lipid content, lipid class composition and total, phospholipid and neutral lipid fatty acid profiles in unfed larvae during yolk sac utilisation. In addition, we studied the LC-PUFA biosynthesis pathway by quantifying expression of fatty acyl desaturase and elongase genes. To this end, we cloned and functionally characterized two cDNAs by heterologous expression in yeast, showing them to code for a Δ6 desaturase and Elovl5 elongase, respectively, that could both be involved in the conversion of EPA to DHA. The level of DHA was maintained, but the proportion of EPA declined, and so the DHA:EPA ratio increased in bluefin tuna larvae during yolk sac utilization. Although this could be the result of relative retention of DHA during a period of generally high fatty acid oxidation and utilization, there was also a great increase in desaturase and elongase expression with larval development. This suggests that increased activity of these enzymes is important for the normal development of tuna larvae related to the provision of adequate DHA for the formation of biomembranes, particularly in neural (brain and eye) tissues.

Keywords: Yolk sac larvae, lipid content, lipid classes, fatty acid composition, cDNA, gene expression.

1. Introduction

Scombrid fish comprise species of high commercial value such as bonitos and tunas. One of the most iconic species is the Atlantic bluefin tuna (ABT), *Thunnus thynnus* (Linnaeus 1758), which has relevant ecological, recreational and commercial importance in Atlantic and Mediterranean ecosystems (Rodríguez-Roda, 1964; Rey, 1999; Fromentin and Powers, 2005). The ABT fishery has become a highly profitable activity with the development of the sushi-sashimi market in Japan increasing the demand for high quality fish, stimulating very high prices and, in consequence, increasing regulated and unregulated fishing effort (Fromentin and Powers, 2005). In recent years considerable research effort has been undertaken in order to develop aquaculture of these large pelagic migratory fish species (Wexler et al., 2003; Margulies et al., 2007; Masuma et al., 2008). Determining nutritional requirements, particularly at larval stages, will be a key task (Mourente and Tocher, 2003, 2009).

The importance of lipids for growth and development of fish is based on their key roles as sources of metabolic energy and as essential components of tissue and cell membranes. In most fish species, lipids are favoured as an energy source compared to proteins and carbohydrates, and are characterized by high contents of long-chain polyunsaturated fatty acids (LC-PUFA) essential for cell membrane structure and function. The reserves of lipid in fish eggs are used by the developing embryo and subsequent larvae for energy metabolism, as structural components of biomembranes and precursors of lipid-derived hormones (Sargent, 1995; Wiegand, 1996; Sargent et al., 1989, 2002). Lipid reserves in teleost fish eggs are stored as lipoproteins in yolk reserves and, in some species, as discrete or fragmented oil globules. Yolk lipoproteins contain primarily phospholipids, mainly phosphatidylcholine and phosphatidylethanolamine, showing high levels of polyunsaturated fatty acids (PUFA), particularly the n-3 LC-PUFA, eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA). Tuna eggs contain a single discrete oil globule, comprised of neutral lipids, primarily triacylglycerol (TAG), steryl ester and/or wax ester, rich in both monounsaturated fatty acids (MUFA) and n-3 LC-PUFA (Wiegand, 1996; Ortega and Mourente,

2010). The LC-PUFA have well-established roles in membrane structure and function as well as energy sources during embryonic and early larval development of marine fish (Sargent et al., 2002). The composition and metabolism of lipid and fatty acids during embryogenesis and yolk-sac larvae development can give useful information of the nutritional requirements during early larval stages (Sargent et al., 2002). This approach can provide insights that can be applied in the development of live feed enrichments and/or artificial feeds (Tocher, 2003).

Tuna tissues have a relatively high level of DHA and a high DHA:EPA ratio (Tocher, 2003; Mourente and Tocher, 2009). However, all marine fish studied to date have only very limited ability to biosynthesize LC-PUFA and so have an absolute requirement for dietary EPA and DHA (Tocher, 2003). The fatty acid profile of lipids from eggs of wild-caught tunas shows high levels of DHA, possibly suggesting that tunas may have a high requirement for this fatty acid (Nichols et al., 1998; Mourente and Tocher, 2009; Ortega and Mourente, 2010). Although the lipid biochemistry underpinning the high level of DHA and the high DHA:EPA ratio in tuna is unclear, it has been generally assumed that tuna must selectively accumulate and retain DHA in their tissues (Ishihara and Saito, 1996; Saito et al., 1996; Mourente and Tocher, 2009). Tuna are top predators of the benthic-pelagic trophic web and the concentration of n-3 LC-PUFA in neural tissues is known to be crucial for effective prey capture from the time of first feeding (Bell et al., 1995; Mourente and Tocher, 2009).

Long-chain PUFA can be biosynthesized from short-chain (C_{18}) PUFA in reactions catalysed by fatty acyl desaturase (Fad) and elongase (Elovl) enzymes. Understanding of the biochemical and molecular mechanisms of LC-PUFA biosynthesis in fish has advanced in recent years and several Fads have been characterized in various fish species. With the exception of a zebrafish bifunctional Δ6/Δ5 Fad (Hastings et al., 2001) and an Atlantic salmon (*Salmo salar*) Δ5 Fad (Hastings et al., 2005), all have been Δ6 Fads (Zheng et al., 2004, 2005, 2009; Tocher et al., 2006; Gonzales-Rovira et al., 2009). Biochemical studies in fish cell lines (Ghioni et al., 1999; Tocher and Ghioni, 1999) led to the hypothesis that some fish may be unable to biosynthesize LC-

PUFA because they lack specific genes in the pathway (Leaver et al., 2008). However, although Δ6 Fads cDNAs have been cloned from all fish species studied so far, and all showed significant activity in heterologous yeast expression systems, Δ6 Fad expression and activity are very low in Atlantic cod (*Gadus morhua*) tissues compared to expression and activity of Δ6 Fad in salmon tissues (Tocher et al., 2006). Elongase cDNAs have been cloned from freshwater species, zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*) and tilapia (*Oreochromis nilotica*); salmonids, Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*); and marine species, Atlantic cod (*Gadus morhua*), turbot (*Psetta maxima*), gilthead sea bream (*Sparus aurata*), cobia (*Rathycentron canadum*) and Southern bluefin tuna (*Thunnus maccoyii*) (Agaba et al., 2004, 2005; Hastings et al., 2005; Zheng et al., 2009; Gregory et al., 2010).

In the present study, we investigated lipid and fatty acid metabolism during early larval development of ABT. Specifically, we describe the changes in total lipid content, lipid class composition and total, phospholipid and neutral lipid fatty acid profiles in unfed ABT larvae during yolk sac utilisation. Moreover, we studied the LC-PUFA biosynthesis pathway by quantifying expression of Fad and Elovl genes. To this end, we cloned and functionally characterized the cDNAs for these genes by heterologous expression in yeast, showing them to be a Δ6 Fad and Elovl5, respectively. The results were discussed in relation to the maintenance of DHA levels and the evolution of DHA:EPA ratios in tuna tissues, and the physiological role for these genes in ABT development.

2. Materials and Methods

2.1 Atlantic bluefin tuna larvae

Atlantic bluefin tuna eggs came from a broodstock composed of 35 fish with an estimated mean body weight of 100 kg. The broodstock were kept in captivity for 3 years in a floating cage located at El Gorguel, off Cartagena coast, SE Spain and were fed on frozen fish, mainly mackerel.

On the 26th and 27th June 2009, 15 captive-reared ABT broodstock fish were induced to mature and reproduce by means of GnRHa implants, and an egg collector, 500 μm mesh screen size, was placed around the cage. Spawning started on June 29th and ceased on July 15th. Over 126 million eggs were collected, with a hatching rate of 84.4% ±13.5%.

Collected eggs were transported in a 500 l plastic tank supplied with pure oxygen to the IEO – Mazarrón aquaculture facilities and placed in 100 L tanks with gentle oxygenation and flow through sterilized sea water in order to clean them. After 1 h, aeration and water flow were stopped to separate buoyant (viable) from non-buoyant (non-viable) eggs, and fertilized eggs collected and incubated at an initial egg density of 1100 eggs per L in 1000 L cylindro-conical tanks. Incubation was carried out at 25-26 ºC, 37 ppt and continuous photoperiod, with a light intensity of 100 lux. An upwelling flow-through, with gentle aeration in order to keep oxygen levels next to saturation, was employed.

2.2 Sample collection and dry weight determination

As described above, the larvae were obtained from the first ever spawning of captive-reared ABT broodstock as part of the EU FP7 project "SELFDOTT". Due to the unique and extremely limited nature of this resource, most larvae were retained for the first research into larval rearing (juvenile production) of this species and so only unfed larvae were available for the present study. Eggs hatched 9th July (0 days post-hatch, 0 DPH) and the larvae were maintained in the conditions described above. Hatching rate in this batch was close to 94 %, and survival of hatched larvae after 1, 2, 3 and 4 days was 69 %, 46 %, 12 % and 4 %, respectively. Thus, the final sample was obtained at 4 days post-hatch (4 DPH) as survival was too low at 5 DPH to obtain viable samples. Each day, three samples of 5-10,000 larvae were collected from the incubator. Water from the incubator was filtered with a 250 μm mesh screen net in order to concentrate the larvae. Larvae were washed with distilled water and excess water blotted through the screen with filter paper. Sampled larvae were collected in cryotubes, frozen in liquid nitrogen and stored at -80ºC until analysis. Replicates of

148 preweighed samples (approximately 50 mg wet weight) were maintained at 110°C for 24 h. The dry
149 weights were determined after cooling *in vacuo* for at least 1 h.
150
151 *2.3 Lipid content, lipid class composition and fatty acid analysis*

152 Total lipid of ABT larvae was extracted from triplicate pooled samples according to the
153 method of Folch et al. (1957). Approximately 1 g of ABT larvae was placed in 20 ml of ice-cold
154 chloroform/methanol (2:1, by vol) and homogenized with an Ultra-Turrax tissue disrupter (Fisher
155 Scientific, Loughborough, U.K.). The non-lipid and lipid layers were separated by addition of 5 ml
156 of 0.88 % (w/v) KCl and allowed to separate on ice for 1 h. The upper non-lipid layer was aspirated
157 and the lower lipid layer dried under oxygen-free nitrogen. The lipid content was determined
158 gravimetrically after drying overnight in a vacuum desiccator.
159 Lipid class composition was determined by high-performance thin-layer chromatography
160 (HPTLC) using 10 x 10 cm plates (VWR, Lutterworth, England). Approximately 2 mg of total lipid
161 was applied as 2 mm streaks and the plates developed in methyl acetate/isopropanol/
162 chloroform/methanol/0.25 % aqueous KCl (25:25:25:10:9, by vol.) to two-thirds up the plate. After
163 desiccation for 20 min, the plate was fully developed with isohexane/diethyl ether/acetic acid
164 (85:15:1, by vol.). The lipid classes were visualized by charring at 160 ^{o}C for 15 min after spraying
165 with 3 % (w/v) aqueous cupric acetate containing 8 % (v/v) phosphoric acid and quantified by
166 densitometry using a CAMAG-3 TLC scanner (version Firmware 1.14.16) (Henderson and Tocher,
167 1992). Scanned images were recorded automatically and analyzed by computer using winCATS
168 Planar Chromatography Manager (version 1.2.0).
169 Fatty acid methyl esters (FAME) were prepared from total lipid, total phospholipids and
170 total neutral lipids by acid-catalyzed transesterification at 50 ^{o}C for 16 h according to the method of
171 Christie (1993). Phospholipids and neutral lipids were separated by TLC, and extraction and
172 purification of FAME was carried out as described by Tocher and Harvie (1988). The FAME were
173 separated and quantified by gas-liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using

a 30m x 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection at 50°C. Hydrogen was used as carrier gas and temperature programming was from 50 °C to 150 °C at 40 °C min^{-1} and then to 230 °C at 2.0 °C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19)

2.4 Cloning of putative fatty acid desaturase and elongase from ABT larvae

Sequences corresponding to the open reading frame (ORF) of *fads* and *elovls* from several fish species were aligned and primers designed on conserved regions (Table 1). GenBank accession numbers of the sequences used in these alignments were AY055749 (*Sparus aurata*), DQ054840 (*Gadus morhua*), AY546094 (*Scophthalmus maximus*), NM_001123575 (*Salmo salar*) and AF301910 (*Oncorhynchus mykiss*) for *Δ6fad*; and GQ204105 (*Thunnus maccoyii*), AY660879 (*Sparus aurata*), AY660881 (*Gadus morhua*), AF465520 (*Scophthalmus maximus*), NM_001123567 and NM_001136552 (*Salmo salar*), DQ067616 (*Oncorhynchus masou*) for *elovl5*-like transcripts. Fragments were obtained by reverse transcription polymerase chain reaction (RT-PCR) (GoTaq® Colorless Master Mix; Promega, Southampton, U.K.) on cDNA synthesised (as described below) from 1 μg of total RNA pooled from 0 to 3 DPH larvae using the conserved region (CR) primers, in the case of elongase, using also primers designed on the sequence of the closely related species *T. maccoyii* (Tm). After sequencing (CEQ-8800 Beckman Coulter Inc., Fullerton, U.S.A.), the ORF fragments were further extended by 3' rapid amplification of cDNA ends (RACE) PCR (FirstChoice® RLM-RACE kit, Ambion, Applied Biosystems, Warrington, U.K.), to obtain the sequence of the 3' UTR. The specific primers used for 3' RACE are shown in Table 1. The *Thunnus thynnus* sequences corresponding to the putative *Δ6fad* and *elovl5* were assembled using BioEdit (Tom Hall, Ibis Therapeutics, CA) and deposited in the GenBank database under accession numbers HQ214238 and HQ214237, respectively.

200 *2.5 Sequence and phylogenetic analysis*

201 The deduced amino acid sequences of the newly cloned ABT *Δ6fad* and *elovl5* cDNAs were
202 aligned with different fatty acyl desaturases and elongases of *Caenorhabditis elegans*, *Mortierella*
203 *alpina*, several fish species, human and mouse (accession numbers shown in Figs 1 and 2) using
204 ClustalW (BioEdit). Phylogenetic analysis was then conducted using the neighbor-joining method
205 (Saitou and Nei, 1987) in MEGA version 4 (Tamura et al., 2007), with confidence in the resulting
206 tree branch topology measured by bootstrapping through 10,000 iterations. Comparison of amino
207 acid and cDNA sequences of ABT and Southern bluefin tuna (SBT, *Thunnus maccoyii*) was
208 performed using BLAST (bl2seq; http://blast.ncbi.nlm.nih.gov/Blast.cgi).
209
210 *2.6 Functional characterisation of cDNAs by heterologous expression in Saccharomyces cerevisiae*

211 PCR fragments corresponding to the ORFs of the putative *Δ6fad* and *elovl5* were amplified from
212 3DPH larvae cDNA using sense and antisense primers containing either a *SacI* or *XhoI* digestion
213 site, respectively (Table 1). In the case of *Δ6fad*, a two stage (semi-nested) PCR had to be
214 performed, first using primers CR-D6DES-F4 and Tt-D6DES-R4 (Table 1), located at the start of
215 the ORF and in the 3' UTR, respectively. For this transcript, PCR was performed using the
216 HotStarTaq DNA Polymerase with Q-solution (Qiagen, West Sussex, U.K.), while the putative
217 *elovl5* ORF was amplified in a single step using the high fidelity PfuTurbo® DNA Polymerase
218 (Stratagene, Agilent Technologies, Cheshire, U.K.), using 35 cycles of amplification with the
219 primers and annealing temperatures shown in Table 1 and following manufacturer's instructions.
220 The DNA fragments were then purified (Illustra GFX™ PCR DNA and gel band purification kit,
221 GE Healthcare Life Sciences, Buckinghamshire, U.K.) and digested with the corresponding
222 restriction endonucleases (New England BioLabs, Herts, U.K.), purified again and ligated into a
223 similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, U.K.). Ligation products
224 were used to transform Top10F' *Escherichia coli* competent cells (Invitrogen) which were screened
225 for the presence of recombinants. The purified plasmids (GenElute™ Plasmid Miniprep Kit, Sigma)

containing the ORFs (confirmed by sequencing) were then used to transform *Saccharomyces cerevisiae* competent cells (S.c. EasyComp Transformation Kit, Invitrogen). Transformation and selection of yeast with recombinant putative *Δ6fad*-pYES2 and *elovl5*-pYES2 plasmids, yeast culture and fatty acid analysis were performed as described in detail previously (Hastings et al., 2001; Agaba et al., 2004; Zheng et al., 2005). Briefly, cultures of recombinant yeast were grown in *S. cerevisiae* minimal medium$^{-uracil}$ supplemented with one of the following faty acid substrates: 18:3n-3, 18:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 or22:4n-6 for Fad; and 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 or 22:4n-6 for Elovl. Fatty acids were added to the yeast cultures at final concentrations of 0.5 (C18), 0.75 (C20) and 2.0 (C22) mM. After 2-days, yeast were harvested, washed, and lipid extracted. The FAME were analyzed by GC and the proportion of substrate fatty acid converted to desaturated or elongated fatty acid product was calculated as [product area/(product area +substrate area)] x 100.

2.7 RNA extraction and quantitative real time PCR (qPCR)

Samples originating from a single pool of larvae collected each day from 0 to 4 DPH were rapidly disrupted in TRI-Reagent (Ambion, Applied Biosystems) using an Ultra-Turrax homogenizer (Fisher Scientific, Loughborough, U.K.). Total RNA was extracted by organic solvent, according to manufacturer's instructions (Ambion, Applied Biosystems), and RNA quality and quantity assessed by electrophoresis (Bioanalyser 2100, Agilent Technologies, Santa Clara, U.S.A.) and spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.). One µg of total RNA per sample was reverse transcribed into cDNA using a Verso^{TM} cDNA kit (ABgene, Surrey, U.K.), following manufacturer's instructions, and using a mixture of random hexamers and anchored oligo-dT (3:1, v/v). Negative controls (containing no enzyme) were performed to later check for genomic DNA contamination.

For quantitative real time PCR (qPCR), primers were designed for both transcripts using the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). In addition, two

252 reference genes, the elongation factor-1α (*elf-1α*; GenBank accession number: FM995222) and *β-*
253 *actin* (GU046791) were quantified (Table 1). Relative quantification was performed in triplicate
254 using a Quantica machine (Techne, Cambridge, U.K.) and the amplification efficiency of each
255 primer pair assessed by serial dilutions of cDNA pooled from the samples being quantified. The
256 amplifications were carried out in a final volume of 20 µl containing 2 µl (for the reference genes)
257 or 5 µl (for larvae samples) diluted (1/20) cDNA, 0.5 µM of each primer and 10 µl AbsoluteTM
258 QPCR SYBR® Green mix (ABgene). In addition, amplifications were carried out with a systematic
259 negative control (NTC-non template control, containing no cDNA). The qPCR profiles contained
260 an initial activation step at 95 °C for 15 min, followed by 30 to 40 cycles: 15 s at 95 °C, 15 s at the
261 annealing Tm and 30 s at 72 °C. After the amplification phase, a melt curve of 0.5 ºC increments
262 from 75 ºC to 90 °C was performed, enabling confirmation of the amplification of a single product
263 in each reaction. The qPCR product sizes and the presence of primer-dimers in the NTC were
264 checked by agarose gel electrophoresis. In addition, qPCR product identities were confirmed by
265 sequencing.
266 Results are given as expression values (obtained from the standard curve performed with cDNA
267 serial dilutions) normalized by the average value of the two reference genes and are expressed as a
268 relative ratio between each sampling day and the expression at 0 DPH.
269
270 *2.8 Materials*

271 The fatty acids, 22:5n−3, 22:4n−6 and 20:4n−3 (all 98–99% pure), were obtained from
272 Cayman Chemical Co. (Ann Arbor, MI, USA), and 18:2n−6, 18:3n−3, 18:4n−3, 18:3n−6, 20:3n−6,
273 20:5n−3 and 20:4n−6 acids (all 99% pure), BHT and chemicals used to prepare the *S. cerevisiae*
274 minimal medium$^{-uracil}$ were from Sigma-Aldrich Co. Ltd. (Poole, UK). Solvents were HPLC grade
275 and from Fisher Scientific (Loughborough, UK).
276
277 *2.9 Statistical analysis*

278 Results are presented as means ± SD (n = 3) except for gene expression data, where a single
279 pool of larvae was analysed. The data were checked for homogeneity of variances using the Bartlett
280 test and, where necessary, arc-sin transformed before further statistical analysis. Differences
281 between mean values were analyzed by one-way analysis of variance (ANOVA), followed when
282 pertinent by a multiple comparison test (Tukey). Differences were reported as statistically
283 significant when $P < 0.05$ (Zar, 1999).
284
285 **3. Results**

286 *3.1 Sequence analyses of ABT larvae desaturase and elongase cDNAs*

287 The overlapping fragments of a putative *fad* and *elovl* that were obtained by classic RT-PCR and
288 3'-RACE-PCR amplification from ABT cDNA were assembled into a sequence of 1817 bp and
289 1170 bp, respectively (gb|HQ214238 and gb|HQ214237, respectively). These sequences included
290 ORFs of 1338 bp for *Δ6fad* and 885 bp for *elovl5*, encoding predicted proteins of 446 and 294
291 amino acids, respectively. Comparison of these sequences with those of *fad* and *elovl* cDNAs
292 (GenBank accession nos. HM032095 and GQ204105, respectively) of the closely related SBT
293 (*Thunnus maccoyii*) revealed practically complete sequence conservation. In fact, both *Δ6fad* and
294 *elovl5* ORF sequences are 99% identical, being translated into a 100% identical Fad protein or 99%
295 identical Elovl5 (Thr in ABT instead of Met in SBT in position 85). The phylogenetic trees of both
296 Δ6 Fad (Fig. 1) and Elovl5 (Fig. 2) also reveal this, with both ABT and SBT protein sequences
297 clustering together. In addition, the ABT putative Δ6Fad clustered closely with other marine fish Δ6
298 Fads, including those of gilthead seabream, European seabass, turbot, cobia and Asian seabass, and
299 more distantly from other fish Fads (including Δ5 Fads and Δ5/Δ6 Fads) and mammalian Δ6 Fads
300 (Fig. 1). The phylogenetic tree constructed for the Elovl proteins (Fig. 2) has clearly separated the
301 vertebrate Elovl5, Elovl2 and Elovl4 proteins, with the ABT and SBT Elovls clustering more

closely with gilthead seabream, turbot and tilapia Elovl5's, followed by salmonid Elovl5 proteins and more distantly with the mammalian Elovl5 proteins.

3.2 Functional characterisation of ABT fatty acyl desaturase and elongase

The fatty acid composition of untransformed yeast *S.cerevisiae* shows four main fatty acids, namely 16:0, 16:1n−7, 18:0 and 18:1n−9 (Hastings et al., 2001), numbered 1-4 in Figs. 3 and 4. When yeast, transformed with the ABT Fad cDNA insert, was grown in the presence of Δ6 desaturase substrate 18:3n-3, two additional peaks were observed in the GC traces, corresponding to the exogenously added fatty acid, 18:3n−3 and its desaturated product, 18:4n−3 (Fig. 3A). The GC traces of Fad-transformed yeast grown in the presence of Δ5 and Δ4 Fad substrates showed peaks for the exogenously added fatty acids, 20:4n−3 and 22:5n−3, but no desaturated products, 20:5n−3 and 22:6n−3, respectively (Figs. 3B and 3C). Identical results were obtained in parallel experiments incubating Fad-transformed yeast with n-6 PUFA, with 18:3n-6 being produced from 18:2n-6, but no products obtained with either 20:3n-6 or 22:4n-6 substrates. In this heterologous assay system, the ABT desaturase showed a preference for the n−3 fatty acid substrates, with approximately 31% of 18:3n−3 converted to 18:4n−3, and 20% of 18:2n−6 converted to 18:3n−6 (Table 2). When yeast, transformed with the ABT Elovl cDNA insert, was grown in the presence of fatty acid substrate for C_{18} elongation, four additional peaks were observed in the GC trace, corresponding to the exogenously added fatty acid, 18:4n−3, its immediate elongated product, 20:4n−3, and the further elongated products, 22:4n−3 and 24:4n−3 (Fig. 4A). The GC trace also showed a large peak of 18:1n−7 (peak 5) indicating elongation of endogenous 16:1n−7, and peaks were also observed that corresponded to 20:1n−9 and 20:1n−7 (peaks 6), indicating elongation of 18:1n−9 and 18:1n−7 (Figs. 4A-C). Similarly, the GC traces of Elovl-transformed yeast grown in the presence of substrate for C_{20} elongation showed additional peaks corresponding to the exogenously added fatty acid, 20:5n−3, its immediate elongation product, 22:5n−3, and the further elongated product, 24:5n−3 (Fig. 4B). Transformed yeast grown in the presence of substrate for C_{22} elongation showed

328 additional peaks corresponding the exogenously added fatty acid, 22:5n−3, and its elongated
329 product, 24:5n−3 (Fig. 4C). Again, similar results were obtained with the corresponding n-6 PUFA
330 substrates. In this heterologous expression system, the ABT elongase showed similar activity
331 towards C_{18} and C_{20} fatty acid substrates, with much lower activity towards C_{22} substrates (Table 2).
332 There was no preference between n−3 and n−6 PUFA substrates for C_{18} and C_{20} elongation, but
333 there may be a slight preference for n−3 with C_{22} elongation (Table 2).
334
335 *3.3 Dry mass, lipid content and lipid class composition of unfed ABT larvae*

336 Yolk sac larvae of ABT showed a significant decrease of around 29 % in dry mass from
337 hatching (DPH0) to DPH4 (Table 3). During the same period, total lipid content decreased by
338 approximately 27 %, on a dry mass basis. The lipid class composition of ABT yolk sac larvae at
339 hatching was predominantly neutral lipids (~77 %), primarily steryl ester/wax ester, TAG and
340 cholesterol, with 23 % total polar lipids, primarily phosphatidylcholine, phosphatidylethanolamine,
341 phosphatidylserine and phosphatidylinositol. The proportion of neutral lipids declined to 59 % by 4
342 DPH due to decreased percentages of both TAG and steryl ester/wax ester. Over the same period,
343 the proportions of membrane lipid increased with total polar lipid and cholesterol rising to 41 % and
344 22 %, respectively (Table 3). Consequently, the nutritional index (TAG/cholesterol) decreased
345 significantly from 1.9 to 0.3.
346
347 *3.4 Fatty acid composition*

348 Total lipids of ABT yolk sac larvae at hatching were characterized by 26 % saturated fatty
349 acids (primarily 16:0, followed by 18:0 and 14:0), 33 % MUFA (primarily 18:1n-9) and 34 %
350 PUFA, primarily the n-3 LC-PUFA, DHA (19 %) and EPA (6 %) with a DHA:EPA ratio of 3.0
351 (Table 4). From hatching to DPH4, the proportion of saturated fatty acids increased to 32 %
352 whereas total MUFA decreased to 27 %, mainly due to increased percentages of 18:0, and
353 decreased percentages of almost all MUFA. There were no significant changes in the proportions of

354 n-3, n-6 or total PUFA during this period of development but the percentages of arachidonic acid
355 (ARA; 20:4n-6) and DHA significantly increased, whereas the proportion of EPA decreased (Table
356 4), such that the DHA:EPA ratio in total lipid increased to 5.0 by DPH4 (Fig.5).

357 Total polar lipids of ABT yolk sac larvae at hatching were characterized by 36 % saturated
358 fatty acids (primarily 16:0 and 18:0), 19 % MUFA (primarily 18:1n-9) and 39 % PUFA, primarily
359 DHA (27 %) and EPA (6 %) with a DHA:EPA ratio of 4.7 (Table 5). There were few major effects
360 of development on polar lipid fatty acid composition from hatching to DPH4, other than a slight but
361 significant increased proportion of total n-6 PUFA, and decreased percentage of EPA (down to 3.7
362 %) with a trend for increased DHA that resulted in the DHA:EPA ratio significantly increasing to
363 7.9 (Table 5, Fig.5). In contrast, there were no overall changes in the proportions of total saturated
364 fatty acids, MUFA or PUFA, including EPA and DHA, in total neutral lipids between DPH0 and
365 DPH4 (Table 6). Similarly, the DHA:EPA ratio was constant at around 2.4-2.5 in neutral lipids
366 (Fig.5).

367

368 *3.5 Expression of Δ6 Fad and Elovl5 elongase in ABT larvae*

369 Quantitative PCR data for each gene were normalized using elongation factor 1α (*elf1α*) and
370 β-actin as reference genes. The expression of *Δ6fad* and *elovl5* elongase were very similar during
371 development (inanition) of ABT yolk sac larvae. There was quantitative increased expresssion of
372 over 8-fold for both genes in only 24 h, from DPH0 to DPH1. In the folowing 24 h the level of the
373 expression remained the same but from DPH2 to DPH4, the expression of *Δ6fad* and *elovl5*
374 increased greatly to attain levels of expression around 23- and 27-fold greater than at DPH0,
375 respectively (Fig.5).

376

377 **4. Discussion**

378

379 The present study is the first report of lipid and fatty acid metabolism in ABT and has focussed
380 specifically on a critical time in marine fish development, such as larvae during the period of yolk
381 sac utilization. Although nutrient utilization and metabolism during early larval development has
382 been a relatively well-studied area in fish there are few comparative data on scombrid species
383 (Weigand, 1996). Fish of the order Perciformes, to which *T. thynnus* (Perciformes, Scombridae)
384 belongs, generally produce eggs with oil globules, showing variability in the egg or oocyte lipid
385 class compositions but generally with neutral lipids predominating over polar lipids (Wiegand,
386 1996). Previously, we have reported lipid and fatty acid compositions of wild ABT eggs (Ortega
387 and Mourente, 2010). The newly-hatched yolk sac larvae obtained from captive ABT showed
388 higher dry mass and total lipid content, and a higher proportion of total neutral lipids, and lower
389 polar lipids, than wild ABT eggs. Importantly, however, the nutritional index (TAG/cholesterol)
390 was almost 50 % lower in yolk sac larvae from the captive ABT than in wild ABT eggs (Ortega and
391 Mourente, 2010). The ABT yolk sac larvae at hatching also showed higher proportions of
392 monoenes and lower proportions of PUFA and a lower DHA:EPA ratio than the total lipid fatty
393 profile of eggs of wild caught ABT (Ortega and Mourente, 2010). These differences in apparent
394 quality parameters (TAG/cholesterol, PUFA level and DHA:EPA ratio) of the larvae from captive
395 ABT compared to eggs from wild ABT suggest that this is an area worthy of future research effort.
396 The decreasing total lipid content, and the decreased proportions of neutral lipids, particularly
397 TAG but also steryl ester/wax ester, showed that lipid was utilized during this early stage of yolk
398 sac larval development in ABT. Phospholipid, TAG or wax ester, exclusively, sequentially or in
399 combination, are all used as energy sources by fish embryos with the pattern varying with species
400 (Sargent et al., 1989, 2002; Wiegand, 1996). Thus, ABT showed a pattern of lipid metabolism
401 during early development similar to that of marine larval fish from temperate waters whose eggs
402 contain high levels of total lipids, including an oil globule, and which preferentially utilize neutral
403 lipids as the primary energy source (Weigand et al., 1996; Sargent et al., 2002). Studies of fatty acid
404 depletion in developing embryos and early larvae of a range of Perciform species have found

405 preferential catabolism of MUFAs along with preferential retention of DHA, ARA and specific
406 saturated fatty acids, usually 16:0 or 18:0 (Mourente and Vazquez, 1996; Wiegand et al., 1996;
407 Mourente et al., 1999; Sargent et al., 2002; Ortega and Mourente, 2010). This reflects the essential
408 structural role of DHA in membranes, the importance of ARA in eicosanoid production and specific
409 roles of saturated fatty acids in the sn-1 position of structural phospholipids.

410 In the present study the ABT larvae were not fed and so were, in effect, starving and the
411 changes in lipids have to be interpreted in that context. Therefore, one explanation for the changes
412 observed in the larval fatty acid composition, showing an increasing DHA:EPA ratio, can be
413 advanced simply in terms of differential oxidation and retention of DHA. The results show total
414 lipid and neutral lipid decreased during the development period and so fatty acids were undoubtedly
415 being utilized for energy. Although it is known that both EPA and DHA can be oxidized in fish, at
416 least salmon, when in dietary excess (Stubhaug et al., 2007), DHA is known to be the fatty acid that
417 is most preferentially retained (Sargent et al., 2002). Whether this is a true active retention or
418 whether it is due to DHA being more slowly and inefficiently oxidized is not clear (Tocher, 2003).
419 The results with the ABT larvae showed that the proportion of polar lipid increased with time and,
420 as polar lipid had a higher DHA content than neutral lipid, the proportion of DHA in total lipid
421 would consequently increase, as observed. In addition however, whereas DHA content in neutral
422 lipid remained constant there was a trend for increased proportions of DHA in polar lipids
423 suggesting that DHA released by hydrolysis of TAG could be then reesterified in polar lipid as
424 observed in previous studies on larval development in marine fish (Tocher et al., 1985; Fraser et al.,
425 1988). So the fact that the content of DHA relative to total fatty acids tends to increase in both total
426 lipid and polar lipid would be consistent with the known phenomenon of retention of DHA.

427 The present study also represents the first investigation of LC-PUFA biosynthesis in ABT and
428 reports the cloning of cDNAs for two important genes in the pathway. Heterologous expression in
429 the yeast *S. cerevisiae* demonstrated that the ABT cDNAs coded for a Δ6Fad and an Elovl5
430 elongase. Alignment and phylogenetic analyses showed that the ABT cDNAs sequences were very

similar to sequences present in the database for Fad and Elovl cDNAs from SBT (Gregory et al., 2010; Schuller et al., 2010). The remarkable level of sequence conservation between the *Thunnus* species was far greater than observed previously between any other teleost species including closely related salmonids (Zheng et al., 2004; Monroig et al., 2010). Furthermore, the functional data obtained with the ABT elongase in the present study were qualitatively identical and quantitatively very similar to the results previously reported for SBT elongase, which was also shown by expression in yeast to be an Elovl5 (Gregory et al., 2010). This is not surprising considering the ORF and translated protein sequences of the *Thunnus* elongases were 99 % identical with only one amino acid difference. The ABT and SBT Fad ORF sequences were also 99 % identical but 100 % identical at the protein level and so, although not functionally characterized, it is certain that the SBT desaturase is also a Δ6Fad as shown for the ABT desaturase in the present study.

The presence of the *Δ6fad* and *elovl5* genes and the role of Δ6Fad and Elovl5 activities in LC-PUFA biosynthesis in marine fish species has been the subject of some discussion and speculation (Zheng et al., 2009; Tocher, 2010). Thus, *Δ6fad* and *elovl5* cDNAs have both been isolated from several marine species including Atlantic cod, turbot, gilthead sea bream, Asian sea bass (*Lates calcarifer*), and cobia (Seiliez et al., 2003; Zheng et al., 2004; Tocher et al., 2006; Mohd-Yusof et al., 2010), all carnivorous, largely piscivorous, species with trophic levels of greater than 3 (Kaushik and Troell, 2010). As such, all these species will normally obtain high levels of EPA and DHA, and rather low levels of 18:3n-3 and 18:2n-6, in their natural diet and so endogenous LC-PUFA biosynthesis from C_{18} precursors is redundant. Consistent with this, LC-PUFA synthesis in hepatocytes or enterocytes from marine species including cod, and European and Asian sea bass, is very low (Mourente et al., 2005; Tocher et al., 2006; Mohd-Yusof et al., 2010). However, the role of Δ6 Fad in the pathway from EPA to DHA (desaturation of C_{24} PUFA intermediates), along with the high expression of *Δ6fad* and *elovl5* in brain in cod, cobia and Asian sea bass, has suggested an alternative role for these enzymes in marine fish, based on a requirement to maintain membrane DHA levels, particularly in neural tissues at times of high demand such as embryonic and larval

457 development (Zheng et al., 2005, 2009; Tocher et al., 2006; Mohd-Yusof et al., 2010). The data
458 from the present study in ABT showing increased expression of *Δ6fad* and *elovl5* during yolk sac
459 larval development is consistent with this hypothesis. Interestingly, the Elovl2 elongase, which has
460 high activity towards C_{22} PUFA would be the more important Elovl in this pathway for production
461 of C_{24} PUFA intermediates, but Elovl2 has only been isolated from Atlantic salmon and not from
462 any marine fish (Morais et al., 2009). However, Elovl5 enzymes in fish have activity towards C_{22}
463 PUFA (Zheng et al., 2004, 2009; Mohd-Yusof et al., 2010), in contrast to mammalian Elovl5 that
464 has very low or no activity towards C_{22} PUFA (Leonard et al., 2000; Inagaki et al., 2002).
465 The above data also offer a further possible explanation for the increasing DHA:EPA ratio
466 observed in total lipid in the ABT larvae. As this increasing ratio was due soley to changes in the
467 DHA:EPA ratio in polar lipid and not neutral lipid, this may suggest that there is an active
468 conversion of EPA to DHA. The *fad* and *elovl* gene expression data are consistent with this, but it is
469 not possible to confirm this from the data available. It is probable that the situation is one where
470 both mechanisms could contribute to the effect. As the larvae were starving in the present study, it
471 is likely that the observed changes in DHA:EPA ratio were more the consequence of retention of
472 DHA through relatively lower oxidation and channeling towards reesterification in polar lipid. In
473 natural development with exogenous feeding, the increased expression of *fad* and *elovl* genes and
474 consequent increased conversion of EPA to DHA would be an important pathway to help satisfy the
475 high requirement for DHA necessary for the rapid development of neural tissue (brain and eye) at
476 this time (Mourente, 2003). It was previously speculated that the high DHA:EPA ratio in tuna
477 tissues in general was consistent with selective metabolism of EPA, through both conversion to
478 DHA via elongation and desaturation and also by catabolism via β-oxidation (Tocher, 2003).
479 In conclusion, the data in the present study with ABT larvae show that the increasing DHA:EPA
480 ratio is most likely the result of retention of DHA in polar lipids and selective catabolism of EPA
481 although the increasing expression of the genes of LC-PUFA biosynthetic pathway, *Δ6fad* and
482 *elovl5*, offers an alternative mechanism. Irrespective of the primary mechanism for increased

DHA:EPA ratio in the present study with unfed larvae, the increasing expression of *Δ6fad* and *elovl5* suggests that endogenous biosynthesis of DHA may be important during normal early development in ABT larvae.

Acknowledgements

The authors would like to thank Dr. David Mazurais from IFREMER, Technôpole Brest-Iroise, for kindly supplying the sequence for the southern bluefin tuna *elf-1α* qPCR primers. The tuna larvae samples were obtained from research partly funded by the EU FP7 project 212797 "From capture based to self-sustained aquaculture and domestication of bluefin tuna, *thunnus thynnus* (SELFDOTT)" coordinated by the Instituto Español de Oceanografía, Spain.

References

Ackman, R.G., 1980. Fish lipids. In: Connell, J. J. (Ed.), Advances in Fish Science and Technology: Fishing News Books, Farnham, pp. 83-103.

Agaba, M., Tocher, D.R., Dickson, C., Dick, J.R., Teale, A.J., 2004. A zebrafish cDNA encoding a multifunctional enzyme involved in the elongation of polyunsaturated, monounsaturated and saturated fatty acids. Mar. Biotechnol. 6, 251-261.

Agaba, M.K., Tocher, D.R., Dickson, C.A., Zheng, X., Dick, J.R., Teale, A.J., 2005. Cloning and functional characterisation of polyunsaturated fatty acid elongases from marine and freshwater teleost fish. Comp. Biochem. Physiol. 142B, 342-352.

Bell, M.V., Batty, R.S., Dick, J.R., Fretwell, K., Navarro, J.C., Sargent, J.R., 1995. Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). Lipids 30, 443-449.

Christie, W.W., 1993, Preparation of derivatives of fatty acids for chromatographic analysis. In: Christie, W.W. (Ed.), Advances in Lipid Methodology-Two. The Oily Press, Dundee. pp. 69-111.

509 Folch, J., Lees, M., Sloane-Stanley, G. H., 1957. A simple method for the isolation and purification
510 of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.
511 Fraser, A.J., Gamble, J.C., Sargent, J.R., 1988. Changes in lipid content, lipid class composition and
512 fatty acid composition of developing eggs and unfed larvae of cod (*Gadus morhua*). Mar. Biol.
513 99, 307-313.
514 Fromentin, J. M., Powers, J. E., 2005. Atlantic bluefin tuna: population dynamics, ecology, fisheries
515 and management. Fish Fisheries 6, 281-306.
516 Ghioni, C., Tocher, D.R., Bell, M.V., Dick, J.R., Sargent, J.R., 1999. Low C18 to C20 fatty acid
517 elongase activity and limited conversion of stearidonic acid, 18:4n-3, to eicosapentaenoic acid,
518 20:5n-3, in a cell line from the turbot, *Scophthalmus maximus.* Biochim. Biophys. Acta 1437,
519 170-181.
520 González-Rovira, A., Mourente, G., Zheng, X., Tocher, D.R., Pendon, C., 2009. Molecular and
521 functional characterization and expression analysis of a Δ6 fatty acyl desaturase cDNA of
522 European sea bass (*Dicentrarchus labrax* L.). Aquaculture 298, 90-100.
523 Gregory, M.K., See, V.H.L., Gibson, R.A., Schuller, K.A., 2010. Cloning and functional
524 characterisation of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*). Comp.
525 Biochem. Physiol. 155B, 178-185.
526 Hastings, N., Agaba, M., Tocher, D.R., Leaver, M.J., Dick, J.R., Sargent, J.R., Teale, A.J., 2001. A
527 vertebrate fatty acid desaturase with Δ5 and Δ6 activities. Proc. Natl. Acad. Sci. U.S.A. 98,
528 14304-14309.
529 Hastings, N., Agaba, M.K., Tocher, D.R., Zheng, X., Dickson, C.A., Dick, J.R., Teale, A.J., 2005.
530 Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs
531 involved in the production of eicosapentaenoic and docosahexaenoic acids from α-linolenic acid
532 in Atlantic salmon (*Salmo salar*). Mar. Biotechnol. 6, 463-474.
533 Henderson, R.J., Tocher, D.R., 1992. Thin layer chromathography. In: Hamilton, R.J., Hamilton, S.,
534 (Eds.), Lipid analysis: a practical approach: IRL Press, Oxford, pp. 65–111.

535 Inagaki, K., Aki, T., Fukuda, Y., Kawamoto, S., Shigeta, S., Ono, K., Suzuki, O., 2002.
536 Identification and expression of a rat fatty acid elongase involved the biosynthesis of C18 fatty
537 acids. Biosci. Biotechnol. Biochem. 66, 613-621.
538 Ishihara, K., Saito, H., 1996. The docosahexaenoic acid content in the lipid of juvenile bluefin tuna
539 *Thunnus thynnus* caught in the sea ofthe Japanese coast. Fish. Sci. 62, 840–841.
540 Kaushik, S., Troell, M., 2010. Taking the fish-in fish-out ratio a step further. Aquacult. Europe 35,
541 15-17.
542 Leaver, M.J., Bautista, J.M., Björnsson, T., Jönsson, E. Krey, G., Tocher, D.R., Torstensen, B.E.,
543 2008. Towards fish lipid nutrigenomics: current state and prospects for fin-fish aquaculture. Rev.
544 Fisheries Sci. 16, 71-92.
545 Leonard, A.E., Bobik, E.G., Dorado, J., Kroeger, P.E., Chuang, L.-T., Thurmond, J.M., Parker–
546 Barnes, J.M., Das, T., Huang, Y.-S., Murkerji, P., 2000. Cloning of a human cDNA encoding a
547 novel enzyme involved in the elongation of long-chain polyunsaturated fatty acids. Biochem. J.
548 350, 765-770.
549 Margulies, D., Suter, J.M., Hunt, S.L., Olson, R.J., Scholey, V.P., Wexler, J.B., Nakazawa, A.,
550 2007. Spawning and early development of captive yelowfin tuna (*Thunnus albacares*). Fish.
551 Bull. 105, 249-265.
552 Masuma, S., Miyashita, S., Yamamoto, H., Kumai, H., 2008. Status of bluefin tuna farming,
553 broodstock management, breeding and fingerling production in Japan. Rev. Fisheries Sci. 16,
554 411-416.
555 Mohd-Yusof, N.Y., Monroig, Ó., Mohd-Adnan, A. Wan, K.-L., Tocher, D.R. (2010) Investigation
556 of highly unsaturated fatty acid metabolism in the Asian sea bass, *Lates calcarifer*. Fish Physiol.
557 Biochem. 36, 827-843.
558 Monroig, Ó., Zheng, X., Morais, S., Leaver, M.J., Taggart, J. B., Tocher, D.R., 2010. Multiple fatty
559 acyl desaturase (Fad) genes in Atlantic salmon: cloning and functional expression of cDNAs
560 confirm presence of three Δ6 Fads. Biochim. Biophys. Acta 1801, 1072-1081.

561 Morais, S., Monroig, Ó., Zheng, X., Leaver, M.J., Tocher, D.R., 2009. Highly unsaturated fatty acid
562 synthesis in Atlantic salmon: characterisation of Elovl5- and Elovl2-like elongases. Mar.
563 Biotechnol. 11, 627-639.
564 Mourente, G., 2003. Accumulation of DHA (docosahexaenoic acid; 22:6n-3) in larval and juvenile
565 fish brain. In: Browman, H., Skiftesvik, A.B. (Eds.), The Big Fish Bang: Institute of Marine
566 Research, Bergen, pp. 239-248.
567 Mourente, G., Tocher, D.R., 2003. An approach to study the nutritional requirements of the bluefin
568 tuna (*Thunnus thynnus thynnus* L.). Cahiers Options Méditerranéennes 60, 143-150.
569 Mourente, G., Tocher, D.R., 2009. Tuna nutrition and feeds: current status and future perspectives.
570 Rev. Fisheries Sci. 17, 374-391.
571 Mourente, G., Vazquez, R., 1996. Changes in the content of total lipid, lipid classes and their fatty
572 acids of developing eggs and unfed larvae of the Senegal sole, *Solea senegalensis* Kaup. Fish
573 Physiol. Biochem. 15, 221–235.
574 Mourente, G., Dick, J.R., Bell, J.G., Tocher, D.R., 2005. Effect of partial substitution of dietary fish
575 oil by vegetable oils on desaturation and oxidation of [1-^{14}C] 18:3n-3 and [1-^{14}C]20:5n-3 in
576 hepatocytes and enterocytes of European sea bass (*Dicentrarchus labrax* L.). Aquaculture 248,
577 173-186.
578 Mourente, G., Rodriguez, A., Grau, A., Pastor, E., 1999. Utilization of lipids by (*Dentex dentex* L.)
579 Osteichthyes, Sparidae larvae during lecitotrophia and subsequent starvation. Fish Physiol.
580 Biochem. 21, 45–58.
581 Nichols, P.D., Virtue, P., Money, B.D., Elliot, N.G., Yearsley, G.K., 1998. Seafood the good food:
582 The oil (fat) content and composition of Australian commercial fishes, shellfishes, and
583 crustaceans. Hobart, Australia: CSIRO Marine Reseach.
584 Ortega, A., Mourente, G., 2010. Comparison of the lipid profiles from wild caught eggs and unfed
585 larvae of two scombroid fish: northern bluefin tuna (*Thunnus thynnus* L., 1758) and Atlantic
586 bonito (*Sarda sarda* Bloch, 1793). Fish Physiol. Biochem. 36, 461-471.

587 Rey, J.C., 1999. Migrationes entre el Atlantico y el Mediterraneo a traves del estrecho de Gibraltar
588 y consideraciones hydrologicas. Biol. Mar. Medit. 6, 220–222.
589 Rodríguez-Roda, J., 1964. Biología del atún, *Thunnus thynnus* (L.), de la costa sudatlántica de
590 España. Inv. Pesq. 25, 33-146.
591 Saito, H., Ishihara, K., Murase, T., 1996. Effect of prey lipids on the docosahexaenoic acid content
592 of total fatty acids in the lipids of *Thunnus albacares* yellowfin tuna. Biosci. Biotechnol.
593 Biochem. 60, 962–965.
594 Saitou, N., Nei, M., 1987. The neighbor-joining method. A new method for reconstructing
595 phylogenetic trees. Mol. Biol. Evol. 4, 406-425.
596 Sargent, J.R., 1995. Origin and functions of egg lipids: nutritional implications. In: Bromage, N.R.,
597 Roberts, R.J. (Eds.), Broodstock Management and Egg and Larval Quality: Blackwell Science,
598 London, pp. 353-372.
599 Sargent, J.R., Henderson, R.J., Tocher, D.R., 1989. The lipids. In: Halver, J.E. (Ed.), Fish Nutrition,
600 2nd Edition: Academic Press, Inc., San Diego, pp. 154-218.
601 Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids. In: Halver J.E., Hardy, R.W. (Eds), Fish
602 Nutrition, 3rd Edition: Academic Press, Inc., San Diego, pp. 182–246.
603 Schuller, K.A., Gregory, M.K., See, V.H.L., Gibson, R.A. (2010) Cloning and functional
604 characterization of fatty acyl desaturase and elongase genes from Southern bluefin tuna *Thunnus*
605 *maccoyii*. In: Proceedings XIV International Symposium on Fish Nutrition & Feeding (ISFNF)
606 31 May - 4 June 2010, Qingdao, China, abstract P358, pp. 510.
607 Seiliez, I., Panserat, S., Corraze, G., Kaushik, S., Bergot, R., 2003. Cloning and nutritional
608 regulation of a delta 6-desaturase-like enzyme in the marine teleost gilthead seabream (*Sparus*
609 *aurata*). Comp. Biochem. Physiol. 135B, 449-460.
610 Stubhaug, I., Lie, Ø., Torstensen, B.E., 2007. Fatty acid productive value and β-oxidation capacity
611 in Atlantic salmon tissues (*Salmo salar* L.) fed on different lipid sources along the whole growth
612 period. Aquacult. Nutr. 13, 145-155.

613 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics
614 Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.
615 Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev.
616 Fisheries Sci. 11, 107-184.
617 Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquaculture
618 Res. 41, 717-732.
619 Tocher, D.R., Ghioni, C., 1999. Fatty acid metabolism in marine fish: Low activity of Δ5
620 desaturation in gilthead sea bream (*Sparus aurata*) cells. Lipids 34, 433-440.
621 Tocher, D.R., Harvie, D.G., 1988. Fatty acid composition of the major phosphoglycerides from fish
622 neutral tissues: (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri* L.)
623 and cod (*Gadus morhua* L.) brains and retinas. Fish Physiol. Biochem. 5, 229-239.
624 Tocher, D.R., Fraser, A.J., Sargent, J.R., Gamble, J.C., 1985. Fatty acid composition of
625 phospholipids and neutral lipids during embryonic and early larval development in Atlantic
626 herring (*Clupea harengus* L.). Lipids 20, 69-74.
627 Tocher, D.R., Zheng, X., Schlechtriem, C., Hasting, N., Dick, J.R., Teale, A.J., 2006. Highly
628 unsaturated fatty acid synthesis in marine fish: cloning, functional characterization, and
629 nutritional regulation of fatty acyl Δ6 desaturase of Atlantic cod (*Gadus morhua* L.). Lipids 41,
630 1003-1016.
631 Wexler, J.B., Scholey, V.P., Olson, R.J., Margulies, D., Nakazawa, A., Suter, J.M., 2003. Tank
632 culture of yellowfin tuna, *Thunnus albacares*: developing a spawning population for research
633 purposes. Aquaculture 220, 327-353.
634 Wiegand, M.D., 1996. Composition, accumulation and utilization of yolk lipids in teleost fish. Rev.
635 Fish Biol. Fish. 6, 259-286.
636 Zar, J.H., 1999. Biostatistical Analysis 4th Edition Prentice-Hall, New Jersey.

637 Zheng, X., Ding, Z., Xu, Y., Monroig, O., Morais, S., Tocher, D.R., 2009. Physiological roles of
638 fatty acyl desaturase and elongase in marine fish: Characterisation of cDNAs of fatty acyl Δ6
639 desaturase and Elovl5 elongase of cobia (*Rachycentron canadum*). Aquaculture 290, 122-131.
640 Zheng, X., Seiliez, I., Hastings, N., Tocher, D.R., Panserat, S., Dickson, C.A., Bergot, P., Teale,
641 A.J., 2004. Characterisation and comparison of fatty acyl Δ6 desaturase cDNAs from freshwater
642 and marine teleost fish species. Comp. Biochem. Physiol. 139B, 269-279.
643 Zheng, X., Tocher, D.R., Dickson, C.A., Dick, J.R., Bell, J.G., Teale, A.J., 2005. Highly
644 unsaturated fatty acid synthesis in vertebrates: new insights with the cloning and characterisation
645 of a Δ6 desaturase of Atlantic salmon. Lipids 40, 13-24.

646

646 **Figure legends**

647 **Fig. 1.** Phylogenetic tree comparing ABT D6Fad with other Fad protein orthologues. The tree was
648 constructed using the Neighbour Joining method with MEGA4. The horizontal branch length is
649 proportional to amino acid substitution rate per site. The numbers represent the frequencies (%)
650 with which the tree topology presented was replicated after 10,000 iterations.
651
652 **Fig. 2.** Phylogenetic tree comparing ABT Elovl5 with other Elovl-like protein orthologues. The tree
653 was constructed using the Neighbour Joining method with MEGA4. The horizontal branch
654 length is proportional to amino acid substitution rate per site. The numbers represent the
655 frequencies (%) with which the tree topology presented was replicated after 10,000 iterations.
656
657 **Fig.3.** Functional characterization of the Atlantic bluefin tuna fatty acyl desaturase in transgenic
658 yeast (*Saccharomyces cerevisiae*) grown in the presence of Δ6 substrate 18:3n-3 (A), Δ5
659 substrate 20:4n-3 (B) and Δ4 substrate 22:5n-3 (C). Fatty acids were extracted from yeast
660 transformed with pYES2 vector containing the ORF of the fatty acyl desaturase cDNA as an
661 insert. The first four peaks in all panels are the main endogenous fatty acids of *S. cerevisiae*,
662 namely 16:0 (1), 16:1n-7 (2), 18:0 (3), and 18:1n-9 (4). Vertical axis, FID response; horizontal
663 axis, retention time.
664
665 **Fig.4.** Functional characterisation of Atlantic bluefin tuna fatty acyl elongase in transgenic
666 *Saccharomyces cerevisiae* grown in the presence of fatty acid substrates 18:4n-3 (panel A),
667 20:5n-3 (B), and 22:5n-3 (C). Fatty acids were extracted from yeast transformed with pYES2
668 vector containing the ORF of the fatty acyl elongase cDNA as an insert. Peaks 1-4 are as
669 described in legend to Figure 3. Peak 5 corresponds to 18:1n-7 arising from the elongation of
670 endogenous 16:1n-7 and peaks 6 correspond to 20:1n-9 and 20:1n-7, resulting from the
671 elongation of endogenous 18:1n-9 and 18:1n-7. In each panel the supplemented substrate fatty

672 acid is marked with an asterisk and the remaining named peaks are elongated products. Vertical
673 axis, FID response; horizontal axis, retention time.
674
675 **Fig.5.** The effects of time after hatching (DPH) on the expression of Δ6 fatty acyl desaturase
676 (*D6fad*) and fatty acyl elongase 5 (*elovl5*) mRNAs (upper panel) and the DHA:EPA ratio in
677 total, polar and neutral lipids (lower panel) in unfed yolk sac larvae of Atlantic bluefin tuna.
678 Columns within each lipid class with a different letter are significantly different (ANOVA,
679 Tukey, $p < 0.05$). DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-
680 3).
681
682
683
684

Table 1. Sequence, annealing temperature (Tm) and size of the fragment produced by the primer pairs used for cloning, functional characterization (restriction sites for *SacI* and *XhoI* underlined), and quantitative PCR (qPCR).

Aim	Transcript	Primer	Primer sequence	Fragment	Tm
ORF cloning	*Δ6fad*	CR-D6DES-F4	ATGGGAGGTGGAGGCCA	1134 bp	58ºC
		CR-D6DES-R3	TCCGCTGAACCAGTCGTTGA		
	elovl5	Tm-Elo5-F1	GGTTACACAGCCGCGTTCT	520 bp	58ºC
		CR-Elo5-R2	AGTTCATAACGAACCACCAGAT		
		CR-Elo5-F1	GCTCTACAATCTGGGCCTC	612 bp	58ºC
		Tm-Elo5-R1	GGAAAGCCATTCTGATGCTC		
3'RACE	*Δ6fad*	Tt-D6DES-F2[1]	ACCGCCAACACCAGTACTTC	1032 bp	60ºC
		Tt-D6DES-F3[2]	CCGTGCACTGTGTGAGAAAC	602 bp	60ºC
	elovl5	Tt-Elo5-F2[1]	GGGATGGCTGTACTTCCAAA	479 bp	60ºC
		Tm-Elo5-F5[2]	CCAAATAGGTTACATGGTCACA CTC	464 bp	60ºC
Functional characterization	*Δ6fad*	CR-D6DES-F4	ATGGGAGGTGGAGGCCA	1465 bp	58ºC
		Tt-D6DES-R4	GAAGCAAAGATCATTCCCACA		
		TtD6DESVF	CCCGAGCTCAATATGGGTGGTG GAGGCCAGC	1341 bp	50ºC
		TtD6DESVR	CCGCTCGAGTCATTTATGAAGA TATGCATC		
	elovl5	TtElo5VF	CCCGAGCTCAAAATGGAGACTT TCAATTATAAACTGAACA	885 bp	60ºC
		TtElo5VR	CCGCTCGAGTCAATCCACCCGC AGTTTCT		
qPCR	*Δ6fad*	Tt-qD6DES-F	CCGTGCACTGTGTGAGAAAC	152 bp	60ºC
		Tt-qD6DES-R	CAGTGTAAGCGATAAAATCAG CTG		
	elovl5	Tt-qElo5-F	CCACGCTAGCATGCTGAATA	236 bp	60ºC
		Tt-qElo5-R	ATGGCCATATGACTGCACAC		
	elf-1α	Tt-qEF1-F	CCCCTGGACACAGAGACTTC	119 bp	60ºC
		Tt-qEF1-R	GCCGTTCTTGGAGATACCAG		
	β-actin	Tt-qBACT-F	ACCCACACAGTGCCCATCTA	155 bp	61ºC
		Tt-qBACT-R	TCACGCACGATTTCCCTCT		

[1]Outer and [2]Inner Primers used for 3' RACE with the FirstChoice RLM-RACE kit.

690 Table 2. Functional characterization of Atlantic bluefin tuna (*Thunnus thynnus* L.) fatty acyl
691 desaturase (Fad) and fatty acyl elongase (Elovl) in yeast *Saccharomyces cerevisiae*.

FA substrate	Product	Conversion	Activity
Fad			
18:3n-3	18:4n-3	31.2	Δ6
18:2n-6	18:3n-6	19.7	Δ6
Elovl			
18:4n-3	20:4n-3	56.9	C18→20
	22:4n-3	19.3	C20→22
	24:4n-3	1.4	C22→24
	Total	77.6	
18:3n-6	20:3n-6	54.8	C18→20
	22:3n-6	22.4	C20→22
	24:3n-6	2.1	C22→24
	Total	79.3	
20:5n-3	22:5n-3	61.4	C20→22
	24:5n-3	12.5	C22→24
	Total	73.9	
20:4n-6	22:4n-6	60.0	C20→22
	24:4n-6	7.0	C22→24
	Total	67.0	
22:5n-3	24:5n-3	8.6	C22→24
22:4n-6	24:4n-6	3.3	C22→24

692 Conversion is expressed as a percentage of total fatty acid (FA) substrate converted to
693 desaturated/elongated products.

694

694 Table 3. Dry mass (percentage), total lipid content (percentage live mass and dry mass) and lipid
695 class composition (percentage of total lipid) of Atlantic bluefin tuna (*Thunnus thynnus* L.) unfed
696 yolk sac larvae from 0 to 4 days post hatch (DPH).

	DPH0	DPH1	DPH2	DPH3	DPH4
Dry mass (%)	8.4 ± 1.8 ^{ab}	8.9 ± 0.3 ^{a}	9.8 ± 0.1 ^{b}	8.4 ± 0.3 ^{a}	6.3 ± 0.9 ^{c}
Total Lipid (% live mass)	2.4 ± 0.4 ^{a}	2.3 ± 0.2 ^{a}	2.4 ± 0.1 ^{a}	1.5 ± 0.1 ^{b}	1.3 ± 0.1 ^{b}
Total Lipid (% dry mass)	29.9 ± 2.3 ^{a}	25.9 ± 1.9 ^{ab}	22.8 ± 1.8 ^{b}	19.2 ± 0.3 ^{b}	19.3 ± 0.8 ^{b}
Lipid Class					
Phosphatidylcholine	10.4 ± 0.3 ^{a}	13.4 ± 0.8 ^{bc}	12.1 ± 1.4 ^{ab}	13.9 ± 1.1 ^{bc}	15.9 ± 0.8 ^{c}
Phosphtidylethanolamine	4.6 ± 0.2 ^{a}	7.4 ± 0.3 ^{b}	7.0 ± 0.5 ^{b}	10.0 ± 0.3 ^{c}	11.1 ± 0.3 ^{d}
Phosphatidylserine	2.1 ± 0.4 ^{a}	4.1 ± 0.4 ^{b}	3.7 ± 0.2 ^{b}	5.8 ± 0.2 ^{c}	7.7 ± 0.2 ^{d}
Phosphatidylinositol	2.6 ± 0.1	2.0 ± 1.0	2.6 ± 0.2	2.6 ± 0.1	2.6 ± 0.2
Phosphatidic acid/cardiolipin	1.0 ± 0.3	1.8 ± 0.8	1.0 ± 0.4	1.5 ± 0.2	1.7 ± 0.1
Sphingomyelin	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.2
Lyso-Phosphatidylcholine	0.7 ± 0.1 ^{a}	0.9 ± 0.2 ^{a}	0.5 ± 0.1 ^{b}	1.1 ± 0.2 ^{ab}	0.9 ± 0.3 ^{ab}
Total polar	22.6 ± 1.3 ^{a}	30.7 ± 1.5 ^{b}	27.8 ± 1.6 ^{b}	35.9 ± 1.5 ^{c}	40.8 ± 2.0 ^{d}
Total neutral	77.4 ± 1.2 ^{a}	69.2 ± 1.5 ^{b}	72.2 ± 1.6 ^{b}	64.1 ± 1.5 ^{c}	59.1 ± 2.0 ^{d}
Cholesterol	11.8 ± 0.8 ^{a}	15.2 ± 0.2 ^{b}	16.1 ± 1.6 ^{b}	21.2 ± 0.9 ^{c}	22.2 ± 0.8 ^{c}
Triacylglycerol (TAG)	23.4 ± 1.5 ^{a}	18.9 ± 0.7 ^{b}	19.3 ± 2.2 ^{b}	9.0 ± 1.2 ^{c}	7.3 ± 0.3 ^{c}
Steryl/wax ester	37.2 ± 0.5 ^{a}	29.7 ± 1.2 ^{b}	31.6 ± 1.1 ^{b}	29.1 ± 1.0 ^{b}	23.5 ± 0.6 ^{c}
Free fatty acid	4.7 ± 0.5	5.3 ± 0.9	4.8 ± 1.3	4.7 ± 0.9	6.0 ± 0.5
TAG:Cholesterol	1.9 ± 0.2 ^{a}	1.2 ± 0.0 ^{b}	1.2 ± 0.2 ^{b}	0.4 ± 0.1 ^{c}	0.3 ± 0.0 ^{c}

697 Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different
698 superscript letter are significantly different ($P<0.05$).
699

699 Table 4. Fatty acid composition (percentage of total fatty acids) of total lipid from unfed Atlantic

700 bluefin tuna (*Thunnus thynnus* L.) yolk-sac larvae from 0 to 4 days post hatch (DPH).

Fatty acid	DPH0		DPH1		DPH2		DPH3		DPH4	
14:0	2.5 ± 0.1	a	2.2 ± 0.2	ab	2.0 ± 0.4	abc	1.6 ± 0.1	bc	1.5 ± 0.2	c
16:0	16.8 ± 0.8		18.8 ± 1.0		19.1 ± 0.8		18.7 ± 0.8		19.3 ± 1.4	
18:0	5.6 ± 0.5	c	6.9 ± 0.5	bc	7.4 ± 0.3	bc	9.0 ± 0.6	ab	10.2 ± 1.5	a
Total saturated[1]	25.6 ± 1.4	b	28.6 ± 1.7	ab	29.2 ± 1.2	ab	30.1 ± 1.3	ab	31.9 ± 2.8	a
16:1n-7	4.9 ± 0.5	a	4.3 ± 0.2	a	4.1 ± 0.2	a	3.2 ± 0.2	b	2.9 ± 0.4	b
18:1n-9	18.1 ± 1.4	a	17.8 ± 0.6	b	17.9 ± 0.4	b	16.2 ± 0.4	b	16.1 ± 1.3	b
18:1n-7	3.9 ± 0.8	a	3.1 ± 0.1	ab	3.1 ± 0.1	ab	2.9 ± 0.2	ab	2.6 ± 0.2	b
20:1n-9	3.3 ± 0.4		3.4 ± 0.1		2.9 ± 1.3		3.1 ± 0.1		3.0 ± 0.1	
22:1	2.1 ± 0.3		2.1 ± 0.1		2.0 ± 0.2		1.7 ± 0.1		1.6 ± 0.2	
24:1n-9	0.5 ± 0.2		0.4 ± 0.1		0.4 ± 0.2		0.7 ± 0.5		0.6 ± 0.4	
Total monoenes[2]	33.0 ± 0.7	a	31.2 ± 0.8	a	30.6 ± 1.8	ab	28.0 ± 1.1	b	27.0 ± 2.3	b
18:2n-6	2.0 ± 0.2	a	1.6 ± 0.1	b	1.5 ± 0.1	b	1.5 ± 0.1	b	1.3 ± 0.0	b
20:4n-6	1.2 ± 0.1	c	1.5 ± 0.0	bc	1.6 ± 0.1	b	2.0 ± 0.2	a	2.2 ± 0.2	a
Total n-6PUFA[3]	4.2 ± 0.1		4.1 ± 0.2		4.2 ± 0.2		4.6 ± 0.3		4.6 ± 0.2	
18:3n-3	0.8 ± 0.1	a	0.7 ± 0.1	ab	0.6 ± 0.1	ab	0.5 ± 0.1	b	0.5 ± 0.1	b
18:4n-3	1.1 ± 0.1	a	1.0 ± 0.0	ab	0.9 ± 0.1	ab	0.7 ± 0.1	bc	0.5 ± 0.2	c
20:4n-3	0.7 ± 0.1	a	0.6 ± 0.0	a	0.6 ± 0.0	a	0.5 ± 0.0	ab	0.4 ± 0.1	b
20:5n-3	6.4 ± 0.3	a	6.2 ± 0.2	a	5.8 ± 0.2	ab	5.1 ± 0.1	bc	4.7 ± 0.4	c
22:5n-3	1.7 ± 0.1		1.7 ± 0.1		1.6 ± 0.1		1.6 ± 0.1		1.5 ± 0.2	
22:6n-3	19.3 ± 0.8	c	20.6 ± 0.5	bc	20.6 ± 0.9	bc	22.7 ± 0.8	ab	23.0 ± 0.9	a
Total n-3PUFA[4]	30.2 ± 1.3		30.8 ± 0.8		30.3 ± 0.8		31.2 ± 0.8		30.8 ± 1.4	
DHA:EPA	**3.0 ± 0.1**	**c**	**3.3 ± 0.0**	**bc**	**3.6 ± 0.3**	**b**	**4.4 ± 0.1**	**a**	**5.0 ± 0.5**	**a**
Total PUFA	34.4 ± 1.3		34.9 ± 1.0		34.5 ± 0.9		35.8 ± 1.0		35.4 ± 1.4	

701 Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different

702 superscript letters are significantly different ($P<0.05$). [1], Totals include 15:0, 20:0 and 22:0; [2],

703 Totals include 16:1n-9 and 20:1n-7; [3], Totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6; [4],

704 Totals include 20:3n-3; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA,

705 polyunsaturated fatty acid.

706

707

707 Table 5. Fatty acid composition (percentage of total fatty acids) of total polar lipids from unfed

708 Atlantic bluefin tuna (*Thunnus thynnus* L.) yolk-sac larvae from 0 to 4 days post hatch (DPH).

Fatty acid	DPH0		DPH1		DPH2		DPH3		DPH4	
14:0	1.6 ± 0.2		1.3 ± 0.2		1.3 ± 0.3		1.2 ± 0.5		1.3 ± 0.1	
16:0	21.1 ± 0.6		22.5 ± 0.2		21.9 ± 2.5		19.5 ± 1.6		19.7 ± 0.0	
18:0	11.7 ± 1.6	b	12.6 ± 0.6	ab	14.1 ± 0.8	a	11.9 ± 0.5	ab	14.0 ± 0.1	a
Total saturated[1]	35.8 ± 1.8	ab	37.3 ± 0.9	ab	38.8 ± 2.9	a	33.5 ± 0.2	b	36.3 ± 0.1	ab
16:1n-7	3.6 ± 1.3		2.6 ± 0.4		2.7 ± 0.8		2.2 ± 0.4		2.6 ± 0.0	
18:1n-9	10.0 ± 1.4		9.3 ± 0.0		9.1 ± 0.3		9.1 ± 0.4		9.5 ± 0.1	
18:1n-7	2.0 ± 0.1		2.3 ± 0.2		2.2 ± 0.2		2.1 ± 0.1		2.1 ± 0.4	
20:1n-9	1.3 ± 0.1		1.3 ± 0.1		1.4 ± 0.1		1.4 ± 0.0		1.4 ± 0.1	
22:1	1.5 ± 0.1		0.5 ± 0.1		0.4 ± 0.0		0.7 ± 0.1		0.4 ± 0.0	
24:1n-9	1.1 ± 0.4		1.5 ± 1.1		1.1 ± 0.5		1.9 ± 1.6		1.3 ± 0.4	
Total monoenes[2]	19.4 ± 3.0		17.7 ± 1.2		17.2 ± 1.2		17.3 ± 1.5		17.3 ± 0.6	
18:2n-6	1.2 ± 0.4		1.0 ± 0.1		1.1 ± 0.2		1.0 ± 0.0		1.1 ± 0.0	
20:4n-6	2.3 ± 0.4		2.1 ± 0.3		2.1 ± 0.1		2.4 ± 0.2		2.6 ± 0.0	
22:5n-6	0.5 ± 0.1	b	0.6 ± 0.1	ab	0.6 ± 0.0	ab	0.7 ± 0.0	a	0.7 ± 0.0	a
Total n-6PUFA[3]	4.4 ± 0.1	bc	4.3 ± 0.1	cd	4.1 ± 0.0	d	4.6 ± 0.0	ab	4.8 ± 0.1	a
18:3n-3	0.3 ± 0.1		0.3 ± 0.0		0.3 ± 0.0		0.2 ± 0.0		0.2 ± 0.0	
18:4n-3	0.4 ± 0.1	a	0.3 ± 0.0	ab	0.2 ± 0.0	b	0.2 ± 0.0	b	0.2 ± 0.0	b
20:4n-3	0.3 ± 0.1		0.3 ± 0.0		0.3 ± 0.1		0.3 ± 0.0		0.2 ± 0.0	
20:5n-3	5.8 ± 0.8	a	5.0 ± 0.7	ab	4.0 ± 0.1	b	4.3 ± 0.5	b	3.7 ± 0.1	b
22:5n-3	1.2 ± 0.1		1.3 ± 0.1		1.3 ± 0.1		1.4 ± 0.0		1.3 ± 0.0	
22:6n-3	26.8 ± 4.1		27.8 ± 4.0		27.2 ± 1.1		30.0 ± 2.5		29.1 ± 1.0	
Total n-3PUFA[4]	34.7 ± 5.1		35.1 ± 4.9		33.4 ± 1.2		36.4 ± 2.9		34.8 ± 1.1	
DHA:EPA	**4.7 ± 0.1**	**e**	**5.5 ± 0.0**	**d**	**6.8 ± 0.0**	**c**	**7.1 ± 0.2**	**b**	**7.9 ± 0.0**	**a**
Total PUFA	39.1 ± 4.9		39.3 ± 4.9		37.5 ± 1.2		41.0 ± 2.9		38.5 ± 1.1	

709 Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different

710 superscript letters are significantly different ($P<0.05$). [1], Totals include 15:0, 20:0 and 22:0; [2],

711 Totals include 16:1n-9 and 20:1n-7; [3], Totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6; [4],

712 Totals include 20:3n-3; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA,

713 polyunsaturated fatty acid.

714

715

715 Table 6. Fatty acid composition (percentage of total fatty acids) of total neutral lipids from unfed

716 Atlantic bluefin tuna (Thunnus thynnus L.) yolk-sac larvae from 0 to 4 days post hatch (DPH).

Fatty acid	DPH0		DPH1		DPH2		DPH3		DPH4	
14:0	3.3 ± 0.1		3.3 ± 0.1		3.2 ± 0.3		3.3 ± 0.2		3.5 ± 0.2	
16:0	14.5 ± 0.8		15.5 ± 0.6		15.5 ± 0.1		15.0 ± 1.2		14.3 ± 0.4	
18:0	4.2 ± 0.4	c	5.6 ± 0.1	a	5.7 ± 0.3	a	5.8 ± 0.1	a	4.9 ± 0.0	b
Total saturated[1]	22.8 ± 1.6		25.1 ± 0.6		25.3 ± 0.3		24.8 ± 1.4		23.6 ± 0.8	
16:1n-7	6.1 ± 0.1	ab	6.1 ± 0.1	ab	6.2 ± 0.1	ab	5.8 ± 0.0	b	6.5 ± 0.4	a
18:1n-9	22.7 ± 1.3	ab	23.6 ± 0.5	a	24.6 ± 0.5	a	23.3 ± 0.5	ab	21.5 ± 0.4	b
18:1n-7	3.1 ± 0.0		3.5 ± 0.5		3.2 ± 0.3		3.2 ± 0.1		3.6 ± 0.1	
20:1n-9	3.9 ± 0.1	b	4.5 ± 0.1	a	4.7 ± 0.1	a	4.7 ± 0.1	a	4.1 ± 0.1	b
22:1	2.7 ± 0.1	b	3.1 ± 0.0	a	3.0 ± 0.0	a	3.1 ± 0.0	a	2.8 ± 0.1	b
24:1n-9	1.0 ± 0.8		0.8 ± 0.4		0.8 ± 0.2		0.7 ± 0.1		0.6 ± 0.1	
Total monoenes[2]	39.7 ± 0.6	b	41.7 ± 0.9	a	42.6 ± 0.5	a	41.0 ± 0.6	ab	39.4 ± 0.3	b
18:2n-6	2.0 ± 0.1		1.9 ± 0.0		2.1 ± 0.1		2.0 ± 0.1		2.3 ± 0.3	
20:4n-6	0.6 ± 0.0	c	0.8 ± 0.0	b	0.8 ± 0.1	b	1.0 ± 0.0	a	1.0 ± 0.1	a
Total n-6PUFA[3]	3.4 ± 0.0	b	3.6 ± 0.1	b	3.8 ± 0.4	b	4.0 ± 0.1	b	5.0 ± 0.6	a
18:3n-3	0.9 ± 0.0		0.9 ± 0.0		1.0 ± 0.1		0.9 ± 0.1		0.9 ± 0.0	
18:4n-3	1.5 ± 0.1	a	1.3 ± 0.0	b	1.2 ± 0.0	b	1.3 ± 0.1	b	1.3 ± 0.0	b
20:4n-3	1.0 ± 0.1		0.8 ± 0.0		0.8 ± 0.1		0.8 ± 0.0		0.8 ± 0.1	
20:5n-3	6.0 ± 0.7	a	5.4 ± 0.1	ab	5.0 ± 0.0	b	5.3 ± 0.2	ab	5.9 ± 0.3	ab
22:5n-3	2.0 ± 0.2	a	1.8 ± 0.1	ab	1.6 ± 0.0	b	1.7 ± 0.1	ab	1.8 ± 0.1	ab
22:6n-3	15.2 ± 1.2	a	12.5 ± 0.4	b	11.8 ± 0.0	b	13.6 ± 1.3	ab	14.0 ± 0.5	ab
Total n-3PUFA[4]	26.5 ± 2.3	a	22.8 ± 0.7	ab	21.4 ± 0.1	b	23.6 ± 1.8	ab	24.7 ± 0.9	ab
DHA:EPA	**2.5 ± 0.1**		**2.3 ± 0.1**		**2.4 ± 0.0**		**2.6 ± 0.1**		**2.4 ± 0.0**	
Total PUFA	29.9 ± 2.3	a	26.4 ± 0.6	ab	25.2 ± 0.5	b	27.5 ± 1.8	ab	29.7 ± 0.4	a

717 Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different

718 superscript letters are significantly different ($P<0.05$). [1], Totals include 15:0, 20:0 and 22:0; [2],

719 Totals include 16:1n-9 and 20:1n-7; [3], Totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6; [4],

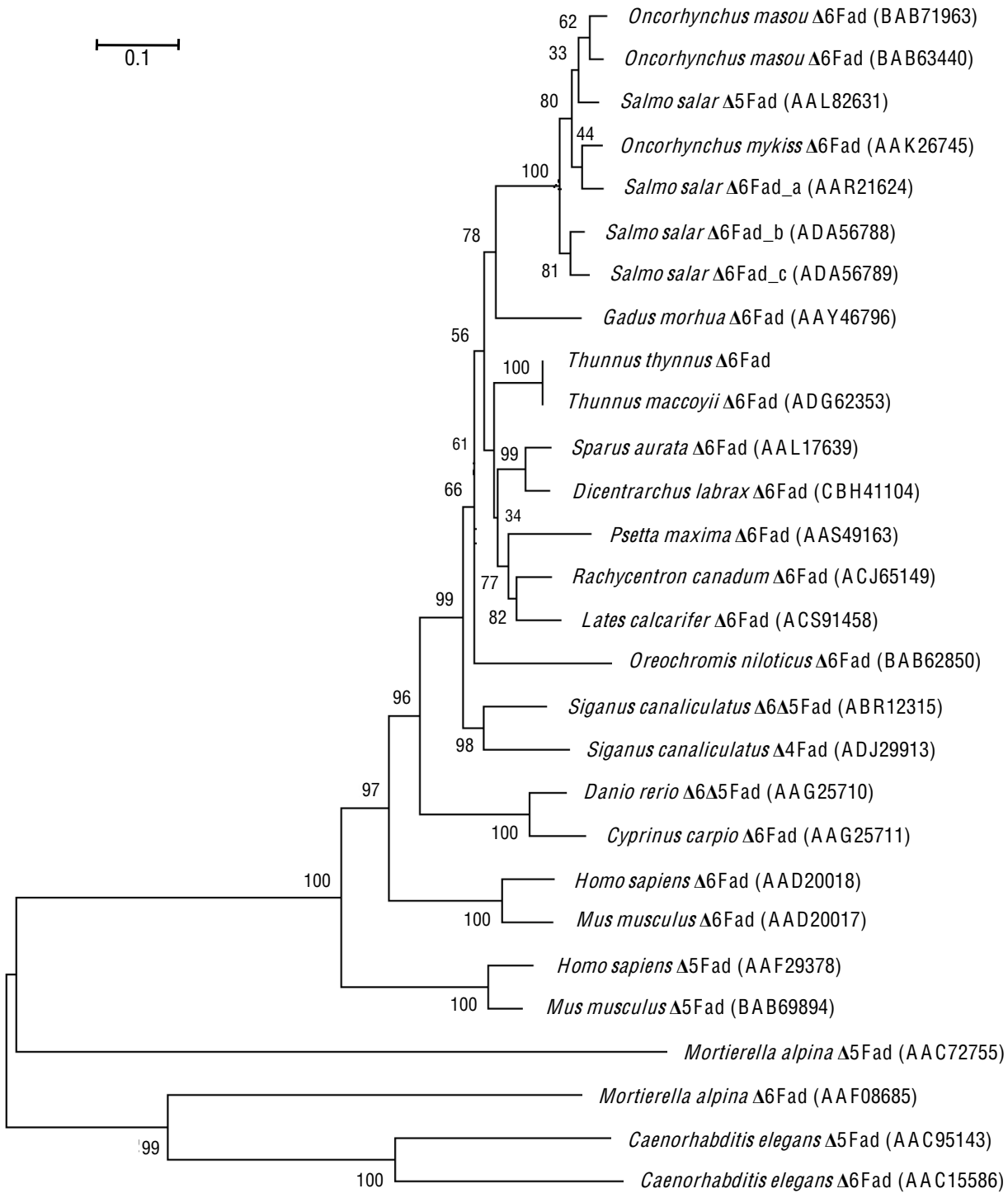
720 Totals include 20:3n-3; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA,

721 polyunsaturated fatty acid.

722

723

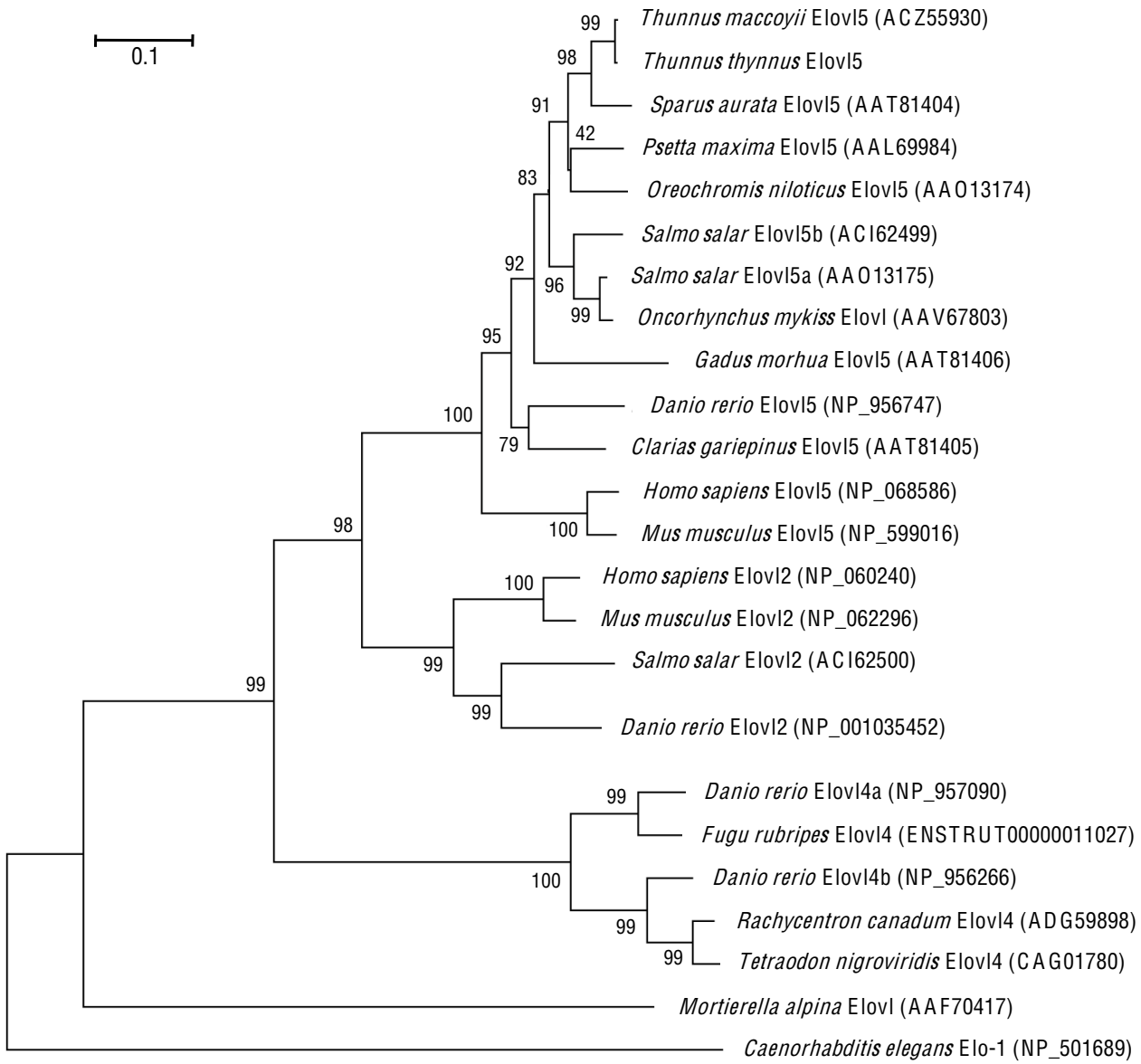
723 Fig.1.



724

725

725 Fig.2.



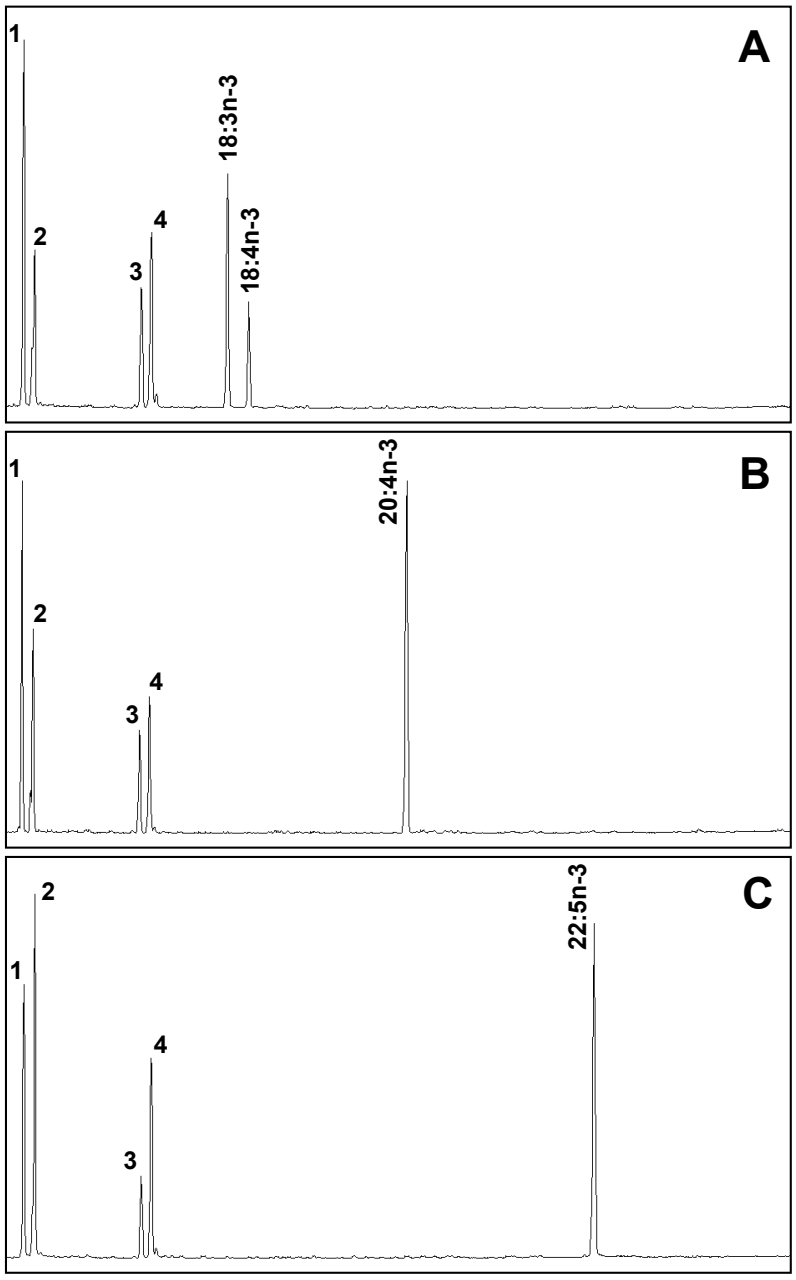
726

727

728 Fig.3.

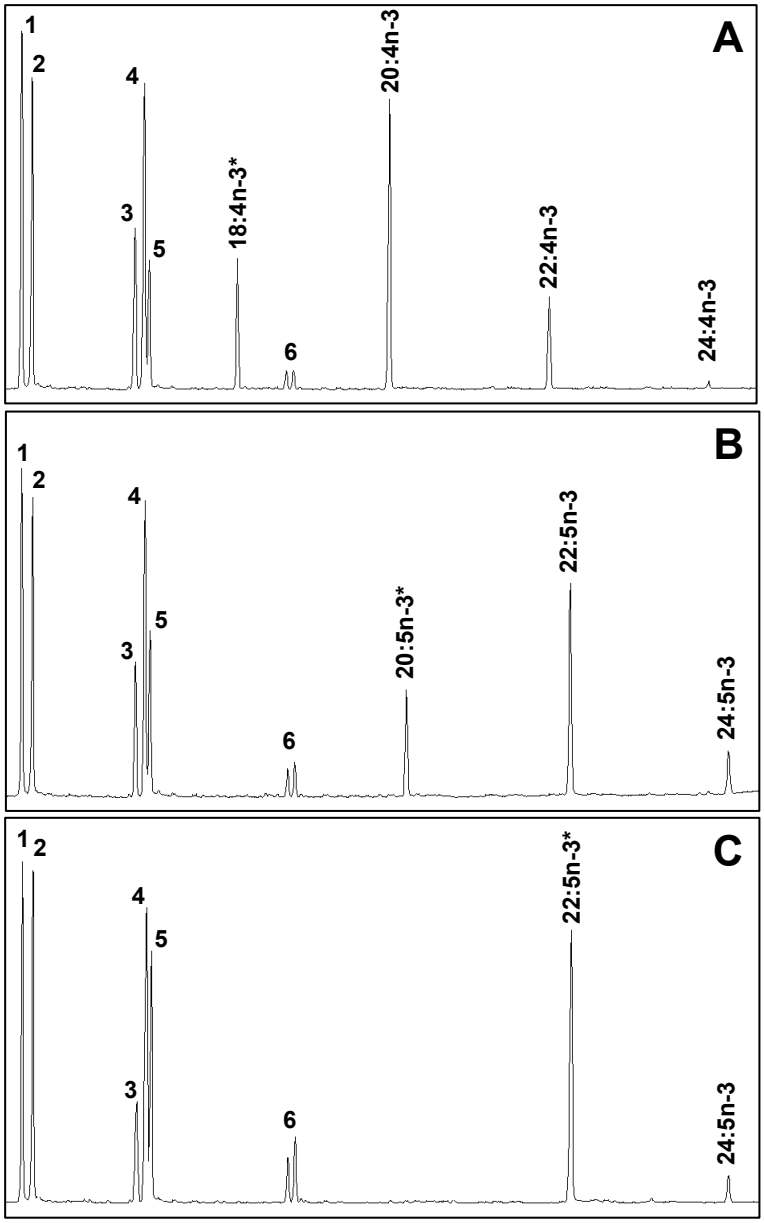
730

732



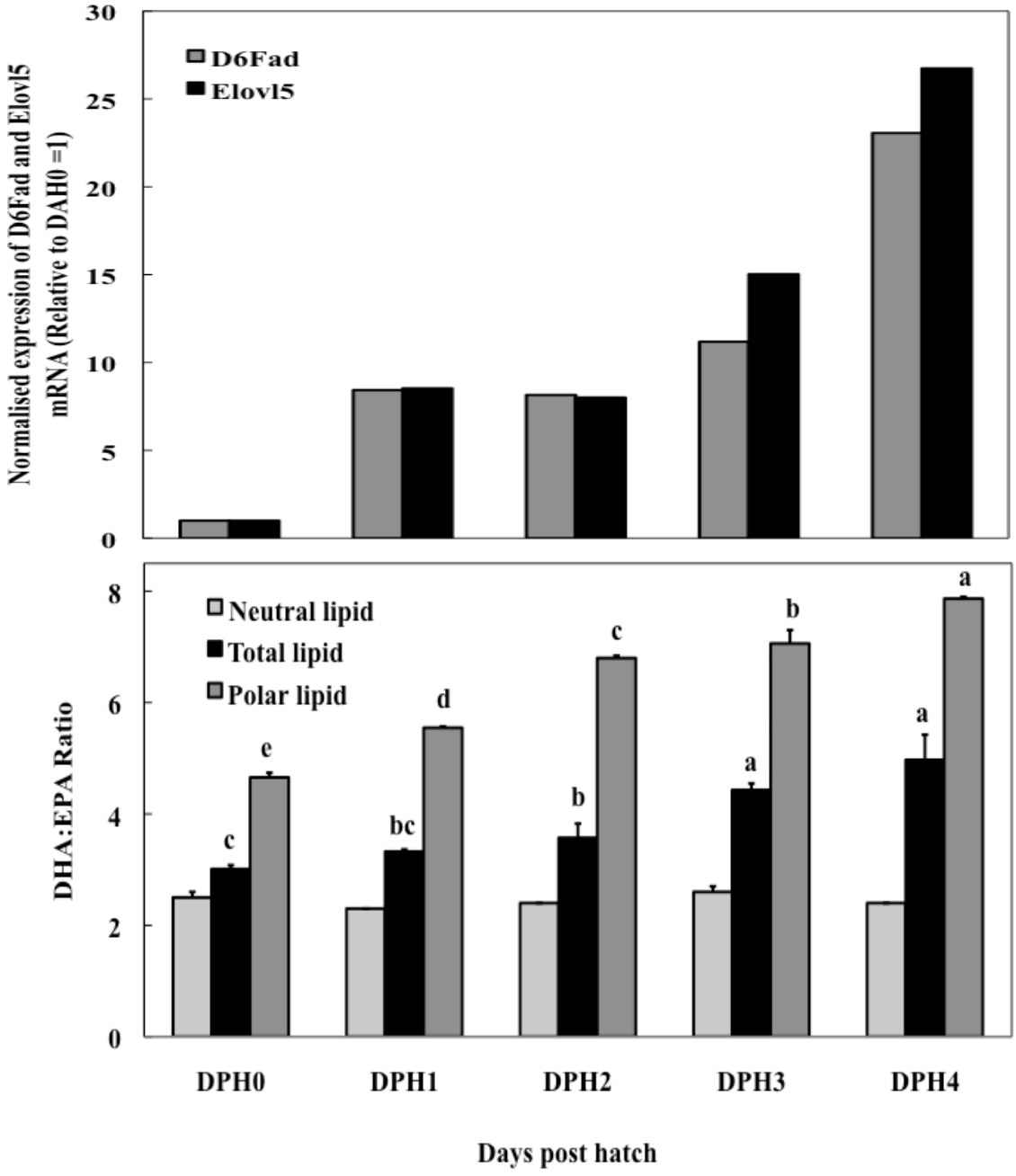
734 Fig.4.

736


A
1
2
3
4
5
6
18:4n-3*
20:4n-3
22:4n-3
24:4n-3
B
1
2
3
4
5
6
20:5n-3*
22:5n-3
24:5n-3
C
1
2
3
4
5
6
22:5n-3*
24:5n-3

737 Fig.5.

739


Normalised expression of D6Fad and Elovl5 mRNA (Relative to DAH0 =1)
D6Fad
Elovl5
30
25
20
15
10
5
0
DHA:EPA Ratio
Neutral lipid
Total lipid
Polar lipid
8
6
4
2
0
c
e
bc
d
b
c
a
b
a
a
DPH0
DPH1
DPH2
DPH3
DPH4
Days post hatch